

## **REMARKS**

### **Rejection under 112, first paragraph**

The promoter genomic elements described in the specification were well-known to the ordinary skilled worker at the time the application was filed. (See, also, Specification, Pages 13-15, where it is described how promoter fragments can be obtained.) For example, the apo C-III gene promoter was described in a number of publications prior to its filing date, including: Reue et al., J. Biol. Chem., 263:6857-6864, 1988 (attached); Leff et al., J. Biol. Chem., 264:16132-16137, 1989 (attached); Ogami et al., J. Biol. Chem., 265:9808-9815, 1990; Fraser et al., J. Biol. Chem., 272:13892-13898, 1997; and Vu-Dac et al., J. Biol. Chem., 272:22401-22404, 1997. Reue et al. (1988) and Ogami et al. (1990) specifically refer to GenBank accessions numbers where sequence information was available.

According to MPEP §2163:

An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention. See, e.g., *Pfaff v. Wells Elecs., Inc.*, 525 U.S. 55, 68, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998); *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406; *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) (one must define a compound by "whatever characteristics sufficiently distinguish it"). "Compliance with the written description requirement is essentially a fact-based inquiry that will 'necessarily vary depending on the nature of the invention claimed.'" *Enzo Biochem*, \*\*>323 F.3d at 963<, 63 USPQ2d at 1613.

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Accordingly, nucleotide sequence information is unnecessary to establish possession of the invention. In *Enzo Biochem. Inc. v Gen-Probe Inc.*, 63 USPQ2d 1609 (Fed. Cir. 2002), the Federal Circuit expressly held that the patentee was not required by §112, first paragraph, to disclose the nucleotide sequences of claimed nucleic acid probes as long as they were able to demonstrate possession of the probes in another way. The court distinguished *Eli Lilly* (see above) because in that case the specification “did not show that the inventors had possession of human insulin cDNA.” The inventors had isolated only the rat cDNA, but tried to claim the human cDNA, when they had not succeeded in cloning it. The issue was not whether they had sequence information, but whether they had any information to establish possession of the gene.

In *Fiers v. Sugano*, 25 USPQ2d 1601 (Fed. Cir. 1993), the party (Fiers) to an interference was trying to establish priority to a gene claim. Since Fiers was not the first to clone the gene, they unsuccessfully argued that they had conceived a method of obtaining it, and that this was adequate to show conception of the gene. In rejecting their argument, the court expressly did not require that they have the gene sequence, as long as they had a physical characteristic of it which would prove they had it in hand. In reaching this conclusion, the court relied on *Amgen Inc. v. Chugai Pharmaceutical Co.*, 927 F.2d 1200, 18 USPQ2d 1016 (Fed. Cir. 1991): “An adequate written description of a chemical invention also requires a precise definition, such as by structure, formula, chemical name, or physical properties, and not merely a wish or plan for obtaining the chemical invention claimed.” As discussed above, promoter genomic DNA of the apo C-III gene was available prior to the filing date. Its nucleotide sequence information is not needed in the present application since the claimed elements were clearly in applicant’s possession.

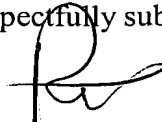
Withdrawal of the rejection is therefore respectfully requested.

In view of the above remarks, favorable reconsideration is courteously requested. If there are any remaining issues which could be expedited by a telephone conference, the Examiner is courteously invited to telephone counsel at the number indicated below.

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The Commissioner is hereby authorized to charge any fees associated with this response or credit any overpayment to Deposit Account No. 13-3402.

Respectfully submitted,



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Richard M. Lebovitz, Reg. No. 37,067  
Attorney for Applicant(s)

MILLEN, WHITE, ZELANO  
& BRANIGAN, P.C.  
Arlington Courthouse Plaza 1, Suite 1400  
2200 Clarendon Boulevard  
Arlington, Virginia 22201  
Telephone: (703) 243-6333  
Facsimile: (703) 243-6410

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## A Regulatory Element in the ApoCIII Promoter That Directs Hepatic Specific Transcription Binds to Proteins in Expressing and Nonexpressing Cell Types\*

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Todd Leff†, Karen Reue‡, Agustin Melian¶, Harry Culver, and Jan L. Breslow||

From the Laboratory of Biochemical Genetics and Metabolism, The Rockefeller University, New York, New York 10021

To better understand the mechanisms that determine cell type-specific gene expression, we have examined the transcriptional activity of a 13-nucleotide long sequence element, designated C3P, located in the promoter of the apoCIII gene. We demonstrate that this element is required for high levels of apoCIII gene expression in hepatic cells and is sufficient to determine hepatic specific expression when introduced into a heterologous promoter. A protein was identified in hepatic cell nuclear extracts, designated AF-1, that binds to this sequence and is presumably responsible for its transcriptional activity in hepatic cells. Even though the C3P element is not active in HeLa cells, a protein with C3P binding specificity was identified in HeLa cell nuclear extracts. While the HeLa protein is similar to the hepatic AF-1 in its binding specificity and relative abundance, it has approximately twice the molecular weight of the hepatic protein, indicating that they are different proteins or different forms of the same protein. A variety of murine tissue types, including those that do not express the apoCIII gene, were found to contain C3P binding proteins. We conclude that the cell type-specific activity of the C3P element is not due to the absence of C3P binding proteins in nonexpressing cells but is the result of qualitative differences in C3P binding proteins in different cell types.

The mechanisms that determine tissue-specific patterns of gene expression are not well understood. The regulation of hepatic specific transcription of the apolipoprotein CIII (apoCIII) gene provides a model for the examination of these mechanisms. The apoCIII gene belongs to the apolipoprotein gene family. These genes code for the major protein components of the lipoprotein transport system that distributes cholesterol and triglycerides throughout the body (reviewed

in Refs. 1-3). *In vivo*, the apoCIII gene is expressed primarily in the liver and to a small extent in the intestine (reviewed in Ref. 1). This cell type-specific pattern of expression is preserved in tissue culture cell lines, where transfected copies of the gene are expressed in HepG2 (hepatic) cells but not in HeLa (epithelial) cells (4). We have recently shown that the apoCIII gene promoter contains at least three regions that together determine transcriptional activity in transfected HepG2 cells (4). The proximal positive element contains a sequence motif (CAGGTGACCTTTG) that is found in the promoters of several apolipoprotein genes (4).<sup>1</sup>

In the current report, we demonstrate that this sequence motif, designated C3P, is essential for high levels of transcription in HepG2 cells and is sufficient to determine hepatic specific expression when inserted into a heterologous promoter. We have also identified a protein in hepatic cells, designated AF-1 (apolipoprotein factor), that binds to the C3P element and is presumably responsible for its activity in hepatic cell types. Surprisingly, a protein was observed in HeLa cell nuclear extracts with the same binding specificity. While the HeLa and hepatic proteins show the same DNA binding specificity and appear to be present in equivalent amounts, gel filtration chromatography demonstrated that the two proteins have distinct molecular weights, indicating that they are different proteins or different forms of the same protein. These results suggest that the differential activity of the C3P element in these two cell types is caused by qualitative differences in their respective C3P binding proteins. Nuclear extracts prepared from a variety of mouse tissues, including liver, were found to contain a C3P binding protein of similar size and relative abundance. We conclude that the cell type-specific activity of the C3P element is due to qualitative differences in the C3P binding proteins in different cell types rather than their absence in nonexpressing cells.

### EXPERIMENTAL PROCEDURES

The construction -821WT contains the apoCIII promoter sequences from -821 to +22 inserted into the CAT expression vector pKT (see Ref. 4 for construction). The mutant apoCIII template -821Xh was constructed by the insertion of a *Xho*I linker into a repaired *Bst*II site at -84 in the wild-type apoCIII promoter. This resulted in the loss of 5 base pairs from -82 to -78 and their replacement by the 8-base pair *Xho*I linker. The vector pXT (see Fig. 3) is derived from the CAT expression vector pCT (5) and contains the adenovirus major late promoter sequences from -50 to +33 (stippled box) linked directly to 60 nucleotides of the SV40 early region noncoding leader sequence (SV40 sequences 5235 to 5175) (striped box). The heterologous promoter plasmids pXT.WTf and pXT.WTr were constructed by inserting a double-stranded oligonucleotide (oligo-WT, see below) in both orientations 60 nucleotides upstream from the start of major late transcription. Plasmids were

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† Investigator of the American Heart Association, New York City affiliate. To whom correspondence should be addressed: The Rockefeller University, Box H29, 1230 York Ave., New York, NY 10021.

‡ Supported by a participating laboratory fellowship from the American Heart Association, New York City affiliate. Present address: Lipid Research Laboratory, V.A. Wadsworth Med. Center, Bldg. 113, Rm. 312, Los Angeles, CA 90024

¶ Fellow of the Stanley J. Sarnoff Society for Cardiovascular Research.

|| Established Investigator of the American Heart Association.

<sup>1</sup> A. Melian, K. Reue, J. L. Breslow, and T. Leff, submitted for publication.

prepared by double banding in cesium chloride and introduced to cells by the calcium phosphate precipitation method as described (4). To avoid possible interactions between apoCIII regulatory elements and other transcriptional regulatory elements, co-transfection of an internal reference plasmid was not used. Instead, each transfection experiment was repeated a minimum of six separate times, with at least two different plasmid preparations. CAT activity was determined by the method of Gorman *et al.* (6).

Nuclear extracts were prepared from tissue culture cells according to Dignam *et al.* (7) and from tissue essentially as described by Gorski *et al.* (8). Human liver tissue was obtained from the Liver Tissue Procurement and Distribution System at the University of Minnesota. DNA binding reactions for the gel mobility shift assay were carried out in a total volume of 10  $\mu$ l and contained 60 mM KCl, 20 mM Hepes<sup>2</sup> (pH 7.9), 4% Ficoll, 1 mM MgCl<sub>2</sub>, and 1  $\mu$ g of poly(dI-dC) (Pharmacia LKB Biotechnology Inc.). In addition each reaction contained 10,000 cpm (about 0.5 ng) of 5'-end-labeled DNA probe and 1  $\mu$ g of nuclear extract protein. Reactions were incubated for 20 min at room temperature and analyzed by electrophoresis on a 5% polyacrylamide gel in 0.25  $\times$  TBE (2.2 mM Tris-borate, 2.2 mM boric acid, 0.5 mM EDTA). Oligonucleotide competitors were added to binding reactions prior to the addition of extract (see figure legends). The sequences of the competitor oligonucleotides are as follows.

Oligo-WT (wild type)	TCGAGCAGGTGACCTTTGCCAGCGCCCTGGG CGTCCACTGGAACGGGTGCGGGACCCAGCT
Oligo-Xh (mutant)	TCGAGCAGCTCTCGAGGCTTTGCCAGCGCCCTGGG CGTGGAGCTCCGAAACGGGTGCGGGACCCAGCT

The relative amount of C3P binding activity in each extract was estimated using the gel mobility shift assay to titrate the amount of protein required to shift 200 pg of the oligo-WT probe to the AF-1 position. All extracts were tested with the same probe preparation.

DNase I footprinting templates were end-labeled as described in the figure legends and incubated with either albumin, as a control, or various amounts of nuclear extract in a 50- $\mu$ l reaction containing 20 mM Hepes (pH 7.9), 60 mM KCl, 1 mM MgCl<sub>2</sub>, 0.5 mM dithiothreitol, 2% polyvinyl alcohol, 10% glycerol, and 1  $\mu$ g of poly(dI-dC). Some extracts contained a phosphatase activity that removed the <sup>32</sup>P label from the templates during the binding reaction. This activity was inhibited by including 0.04 mM ATP in the binding reaction. After 15 min on ice followed by a 3-min incubation at room temperature, 6 ng of DNase I (Worthington) in 50  $\mu$ l of 5 mM CaCl<sub>2</sub>, 1 mM EDTA was added and the incubation continued at room temperature for another 60 s. Digestion was stopped by the addition of 100  $\mu$ l of 0.6 M NaAc, 1% sodium dodecyl sulfate, 20 mM EDTA, and 100  $\mu$ g/ml glycogen. The DNA was phenol-extracted, precipitated with ethanol, and analyzed on a 6% polyacrylamide, 6 M urea sequencing gel. G+A sequencing ladders (9) of probe templates were run as markers.

Chromatography on Superose 12 (HR 10/30 Pharmacia) was carried out at 4 °C at a flow rate of 0.25 ml/min. The column was equilibrated and eluted in a buffer containing 20 mM Hepes (pH 7.9), 200 mM KCl, 1 mM MgCl<sub>2</sub>, 0.1 mM EDTA, 17% glycerol, 5% ethylene glycol, 1 mM dithiothreitol, 0.4 mM phenylmethylsulfonyl fluoride, 2.5  $\mu$ g/ml each of leupeptin, chymostatin, antipain, and pepstatin.

## RESULTS AND DISCUSSION

To characterize the role of the C3P element in determining transcriptional activity of the apoCIII gene, a small mutation in this element was introduced that replaced the five nucleotides between -84 and -78 with an 8-nucleotide *Xho*I linker (referred to as the Xh mutant). The CAT expression vector pKT containing wild-type or mutant apoCIII promoter sequences from -821 to +22 was transfected into HepG2 and HeLa cells. The Xh mutation caused an 8-fold decrease in the transcriptional activity of the apoCIII promoter in HepG2 cells (Fig. 1), indicating that C3P is required for normal hepatic expression of the apoCIII gene. Neither the wild-type nor mutant constructions were expressed in HeLa cells.

To identify the proteins that interact with the C3P element, the wild-type and mutant templates described above were analyzed by the gel mobility shift and DNase I footprinting

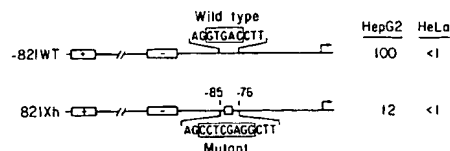


FIG. 1. The C3P element is required for hepatic expression of the apoCIII gene. Transcriptional activity of wild-type (WT) and Xh mutant (Xh) apoCIII promoters in transfected HepG2 and HeLa cells is shown. Constructions containing wild-type or mutant apoCIII promoter sequences (-821 to +22, with respect to the start site of apoCIII transcription) cloned into the CAT expression vector pKT (4), were transfected into HepG2 and HeLa cells. CAT activity is presented relative to the wild-type construction and represents the averages of six experiments. Open boxes indicate previously described (4) positive and negative transcriptional elements in the apoCIII promoter. Boxed sequences represent nucleotides that differ between the wild-type and mutant templates.

assays. A wild-type fragment of the apoCIII promoter (-110 to +22) shows a single major shifted protein band on a mobility shift gel which does not appear when a template containing the Xh mutation is used (Fig. 2A, compare lanes 1 and 2). These results demonstrate that the binding of a protein, designated AF-1 (apolipoprotein factor), correlates with the transcriptional activity of the C3P element. This protein is probably the trans-acting factor that determines the transcriptional activity of the C3P element. The binding of AF-1 to the wild-type template can be competed by the addition of an unlabeled oligonucleotide representing the C3P element but not by the addition of a similar oligonucleotide containing the Xh mutation (Fig. 2A, compare lanes 3 and 4). In addition, an apoCIII template extending to -82 bound the protein, while a template extending to only -77 did not (Fig. 2A, lanes 5 and 6). These results indicate that the sequence between -82 and -77, which includes half of the C3P sequence, is required for AF-1 binding.

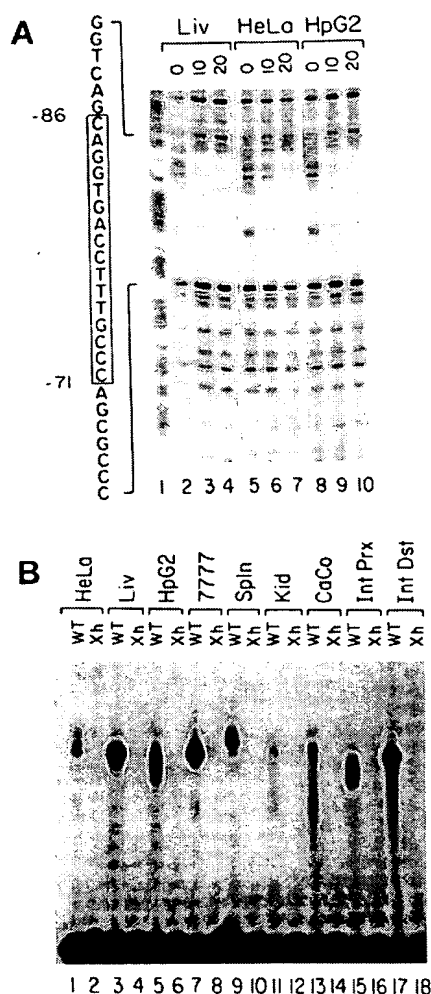
To precisely map the regions of the apoCIII promoter that interact with DNA binding proteins, we carried out DNase I footprinting analysis of a fragment of the apoCIII promoter with proteins from HepG2 nuclear extracts. The results of these experiments indicated that the region between positions -86 and -71 is protected by a DNA binding protein (Fig. 2B). This protection can be competed by the wild-type oligonucleotide (Fig. 2B, lanes 2 and 4) and does not occur on a template containing the Xh mutation (Fig. 2B, lanes 9-11), indicating that the protein detected in the gel mobility shift assay is the same protein responsible for the -86 to -71 footprint.

The inactivity of the apoCIII promoter in HeLa cells makes it difficult to determine whether the C3P element has any effect, positive or negative, on transcription in this cell line. To address this question and to directly evaluate the cell type specificity of the C3P element, we analyzed its effect on transcription in a heterologous promoter construction that is expressed equally well in HepG2 and HeLa cells. An oligonucleotide representing the C3P sequence was inserted 60 nucleotides upstream from the transcriptional start site of the adenovirus major late promoter in the CAT expression vector pXT. The presence of this element caused a 6-fold stimulation of pXT expression in HepG2 cells but had no significant effect on expression in HeLa cells (Fig. 3). These results confirm that the C3P sequence is a hepatic specific transcriptional regulatory element.

Sequence elements similar to C3P appear in the promoters of several apolipoprotein genes (4, 10), including apoB. The observation that the C3P element is active in hepatic but not HeLa cells (Fig. 3) is supported by our recent results demon-

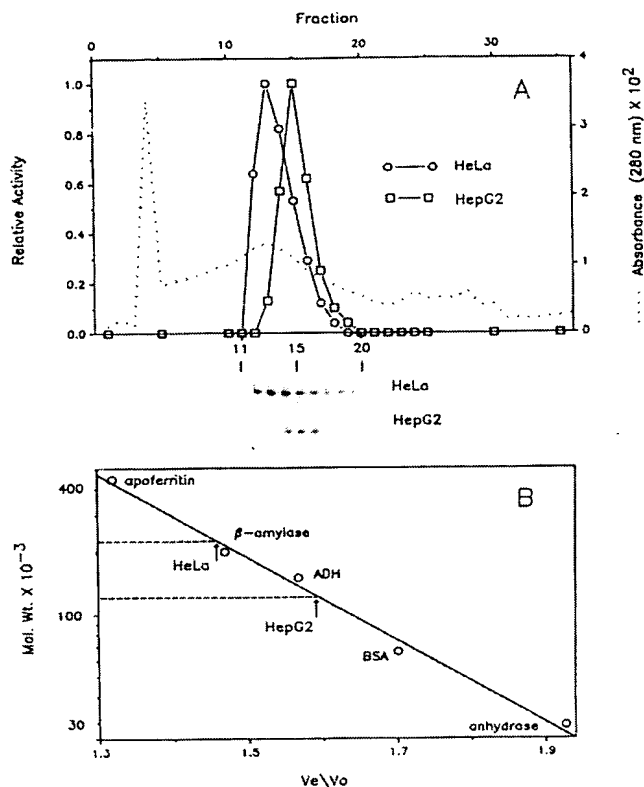
<sup>2</sup> The abbreviation used is: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.





**FIG. 4. C3P binding proteins are present in a variety of cell and tissue types.** A, DNase I footprinting analysis of C3P binding proteins in hepatic and HeLa cell nuclear extracts. DNA templates isolated from wild-type apoCIII promoter and labeled as in Fig. 2 were incubated with mouse liver (Liv), HeLa cell (HeLa), or HepG2 (HpG2) nuclear extracts as indicated and analyzed by DNase I footprinting as described under "Experimental Procedures." Lane 1 is a G+A sequencing ladder of the same template. Boxed nucleotides represent sequences protected from DNase activity in each of the three extracts. B, gel mobility shift assay of C3P binding activity in nuclear extracts prepared from a variety of cell and tissue types. Labeled DNA templates (0.2 ng) were either oligo-WT (WT) or oligo-Xh (Xh) as indicated. Extracts were prepared from HeLa cells (HeLa), mouse liver (Liv), HepG2 (HpG2), rat hepatoma cells RH7777 (7777), mouse spleen tissue (Spln), mouse kidney tissue (Kid), human colonic cell line CaCo-2 (CaCo), mouse proximal intestine tissue (Int Prx), and mouse distal intestine tissue (Int Dst).

that the same protein is present in both cell types but appears only as a dimer in HeLa cells. In this case, the 240-kDa species seen in the HepG2 extract (Fig. 5A, HepG2 gel mobility shift panel) would suggest that a fraction of AF-1 in HepG2 cells is also present as a dimer. The HeLa and hepatic proteins could be products of the same gene but differentially modified to influence their ability to form dimers and their transcriptional activity. These modifications could include phosphorylation or glycosylation, both of which have been shown to occur on transcription factors (for examples see Refs. 11 and 12). Finally, the size differences could be artifacts of



**FIG. 5. C3P binding proteins from HepG2 and HeLa cells have different molecular weights.** A, nuclear extracts prepared from HepG2 cells (0.7 mg) or HeLa cells (1.1 mg) were chromatographed on a Superose 12 column (HR 10/30, Pharmacia) as described under "Experimental Procedures." Fractions were analyzed by the gel mobility shift assay using oligo-WT as the labeled probe. The regions of the mobility shift gels containing the shifted bands are shown below panel A for fractions 11-20. Binding activity (determined by densitometric scanning of mobility autoradiograms) was normalized to the highest amount of activity in each extract. The HepG2 profile represents only the major species seen on the gel mobility shift pattern. B, the column was calibrated by consecutive runs with the following molecular weight markers: apoferritin (443,000),  $\beta$ -amylase (200,000), alcohol dehydrogenase (ADH) (150,000), bovine serum albumin (BSA) (66,000), and carbonic anhydrase (anhydrase) (29,000).  $V_e$  is the elution volume of the protein and  $V_0$  is the void volume of the column. Arrows indicate the location of activity peaks of HeLa and HepG2 extracts as indicated.

extract preparation, and the active forms of the protein in HeLa and HepG2 cells might be identical. In this case, their activities could be influenced by their interaction with proteins unique to that cell type. Purification and characterization of C3P binding proteins from HepG2 and HeLa cells will resolve these different possibilities.

To determine if C3P binding activity is present in other tissues, nuclear extracts were prepared from a variety of cell and tissue types and assayed by the gel mobility shift assay using wild-type and Xh mutant templates. The cell and tissue types tested include those that express the apoCIII gene (liver and intestine) and those that do not (spleen and kidney). The results shown in Fig. 4B demonstrate that every extract tested contained a protein that bound to the wild-type but not to the mutant sequence. In addition, the relative quantity of C3P binding activity in each extract was found to be roughly equivalent (Table I). The C3P binding activity in each of these extracts was further analyzed by DNase I footprinting on the CIII promoter template. Each extract protected the

TABLE I  
Characterization of C3P binding activity in various cell and tissue extracts

Extract source <sup>a</sup>	Molecular mass <sup>b</sup> kDa	Specific activity <sup>c</sup> unit/mg extract
Tissue culture cells		
HepG2	133 ± 28	180
HeLa	240 ± 48	160
Mouse		
Liver	117 ± 23	670
Kidney	110 ± 23	820
Spleen	125 ± 25	— <sup>d</sup>
Proximal small intestine	110 ± 23	700
Distal small intestine	110 ± 23	740
Rat liver	125 ± 23	ND <sup>e</sup>
Human liver	133 ± 27	ND

<sup>a</sup> Tissue extracts were prepared by the method of Gorski *et al.* (8) and tissue culture cell extracts by the method of Dignam *et al.* (7).

<sup>b</sup> Molecular mass of the major form in each extract was determined as described in the legend to Fig. 5. Figures represent the average of two or three runs.

<sup>c</sup> One unit of binding activity is defined as the amount of protein required to shift 200 pg of probe in a standard mobility shift reaction (see "Experimental Procedures").

<sup>d</sup> Spleen extract contained a nuclease or phosphatase activity that made accurate activity measurements by this method impossible.

<sup>e</sup> ND, not determined.

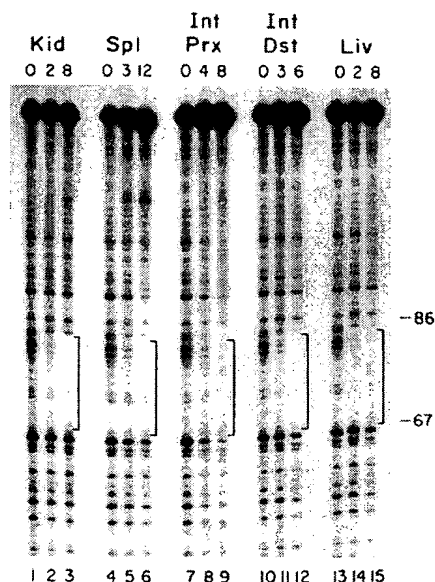


FIG. 6. C3P binding proteins in different tissues give the same footprint. Nuclear extracts were prepared from various mouse tissues and used in DNase I footprinting reactions of the apoCIII promoter. The amount of extract in micrograms is indicated at the top of each lane for liver (Liv), kidney (Kid), spleen (Spl), proximal small intestine (Int Prx), and distal small intestine (Int Dst) nuclear extracts. The template is a fragment of the apoCIII promoter containing sequences from +22 to -220 5'-end-labeled at +22.

same region of the template as the liver AF-1 protein (Fig. 6), suggesting that each tissue contains an equivalent C3P binding activity.

As in the comparison between HepG2 and HeLa cells, the differences in mobility of the C3P binding activity-DNA complexes in the various mouse tissue extracts suggest qualitative differences in their DNA binding proteins. However, in contrast to the HepG2 and HeLa observations, the molecular weights of the C3P binding proteins in each mouse tissue, as determined by the gel filtration chromatography, were

TABLE II  
C3P element sequence similarities in other genes

Gene <sup>a</sup>	Location <sup>b</sup>	Sequence <sup>c</sup>	Ref.
ApoCIII (h)	-86	AGGTGACCTTTG	4
ApoB (h)	-82	AGGcGcCCTTTG	10
ApoB (h)	-69	GACCTTTG	
ApoA1 (h)	-135	AGcTGAtCCTT-G	24
ApoCII (h)	-157	AcGTGACCTT-G	25
$\alpha_1$ -Antitrypsin (h)	-86	gGGTGACCTT-G	18
Myoglobin (h)	+645	gGGTGACCTTTG	26
S-Protein (h)	-136	AtGTGACCTTTG	27
Intestinal FABP (h)	-847	tGGTGACCTTTt	28
Oxytocin-neuro- physin (h)	-165	cGGTGACCTT-G	29
Transthyretin (m)	-1.9 kb	tGaTGACCcTTG	17
$\alpha$ -Fibrinogen (r)	-510	AGGTGACCTTca	30
Parotid secretory protein (m)	-134	gGGTGACCTTTG	31
Acyl-CoA oxidase (r)	-574	AcGTGACCTTTG	32

<sup>a</sup> Criteria for inclusion in the table were that the similar sequence occurred within 2 kilobases of the transcriptional start site on either strand and in a noncoding region of the gene. Species of origin is given in parentheses: h, human; r, rat; m, mouse. FABP, fatty acid binding protein.

<sup>b</sup> Location of the first nucleotide of the sequence shown in the third column relative to the start of transcription.

<sup>c</sup> Sequences are shown 5' to 3'. Matches to the C3P sequence are shown as upper case letters.

similar, between 110,000 and 125,000 (Table I). The significance of the size difference between the human and mouse activities and between expressing and nonexpressing cell types is not understood and can only be investigated by purification and characterization of the proteins involved.

The presence of proteins in different cell types that share DNA binding specificity but differ in transcriptional activity has been observed in other systems. The trans-acting proteins OTF-1 and OTF-2 bind to the same octamer recognition sequence but are distinct proteins with different molecular weights and different activities (13-15). OTF-2 is found only in lymphocytes and activates transcription of some immunoglobulin genes by binding to the octamer sequence in their promoters. OTF-1 is found in a variety of other cell types and while it recognizes the same octamer sequence it does not activate transcription of immunoglobulin genes (13, 16).

The apoCIII gene is a member of the apolipoprotein gene family (3), the products of which are important components of the lipoprotein system that transports triglycerides and cholesterol throughout the body and regulates their levels in the serum (1, 2). Four members of this gene family (including apoCIII) contain C3P sequence homologies in their promoter regions (Table II). We have recently determined that AF-1 interacts with these apolipoprotein genes,<sup>1</sup> and we believe that AF-1 is a major determinant of apolipoprotein gene expression. A search of the GenBank for homologies to the C3P sequence element (CAGGTGACCTTTG) revealed homologies in the promoters of several mammalian genes in addition to the apolipoprotein genes (Table II). The homologies identified in the transthyretin and  $\alpha_1$ -antitrypsin genes are notable in that they reside in transcriptionally active regions of the promoter (17, 18). Cross-competition experiments with an oligonucleotide containing the transthyretin homology indicated that AF-1 can bind to these sequences.<sup>3</sup> A protein has

<sup>3</sup> T. Leff and R. Costa, unpublished observations.



been identified from rat liver that interacts with the  $\alpha_1$ -antitrypsin homology (12), but its relationship to AF-1 has not been determined. Other well characterized genes expressed in the liver, including albumin and  $\alpha$ -fetoprotein, do not contain similar sequences (19–23), suggesting that AF-1 does not interact with these promoters. In addition, oligonucleotides containing the binding site sequences of the transcription factors HNF-1, C/EBP, and HNF-3 (17) did not compete for AF-1 binding sites (data not shown). These results indicate that AF-1 is a distinct protein from these hepatic transcription factors.

Although the transthyretin and  $\alpha_1$ -antitrypsin genes are expressed in the liver as are the apolipoprotein genes, C3P sequence similarities were found in genes that are expressed in a wide variety of tissues (Table II), including pituitary (oxytocin-neurophysin), muscle (myoglobin), parotid (parotid secretory protein), and intestine (fatty acid binding protein). Although the significance of these sequence similarities is difficult to evaluate, they suggest that AF-1 may have a transcriptional role in a variety of tissue types. Purification and characterization of C3P binding proteins from HepG2 and HeLa cells and from different tissues will clarify its cell type-specific transcriptional activity and its role in regulating tissue-specific expression of the apolipoprotein genes.

**Acknowledgments**—We would like to thank Dr. Jonathan Smith for providing the pXT construction and Alexandra McNear and Lorraine Duda for help in preparing the manuscript.

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# Human Apolipoprotein CIII Gene Expression Is Regulated by Positive and Negative Cis-acting Elements and Tissue-specific Protein Factors\*

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Karen Reue†, Todd Leff§, and Jan L. Breslow¶

From the Rockefeller University, New York, New York 10021

Apolipoprotein CIII (apoCIII) is a major protein constituent of triglyceride-rich lipoproteins and is synthesized primarily in the liver. Cis-acting DNA elements required for liver-specific apoCIII gene transcription were identified with transient expression assays in the human hepatoma (HepG2) and epithelial carcinoma (HeLa) cell lines. In liver cells, 821 nucleotides of the human apoCIII gene 5'-flanking sequence were required for maximum levels of gene expression, while the proximal 110 nucleotides alone were sufficient. No expression was observed in similar studies with HeLa cells. The level of expression was modulated by a combination of positive and negative cis-acting sequences, which interact with distinct sets of proteins from liver and HeLa cell nuclear extracts. The proximal positive regulatory region shares homology with similarly located sequences of other genes strongly expressed in the liver, including  $\alpha_1$ -antitrypsin and other apolipoprotein genes. The negative regulatory region is strikingly homologous to the human  $\beta$ -interferon gene regulatory element. The distal positive region shares homology with some viral enhancers and has properties of a tissue-specific enhancer. The regulation of the apoCIII gene is complex but shares features with other genes, suggesting shuffling of regulatory elements as a common mechanism for cell type-specific gene expression.

The apolipoproteins are a class of lipid binding polypeptides which transport cholesterol, triglycerides, and phospholipids in the plasma in the form of lipoprotein particles. In addition to their function in determining lipoprotein structure, some apolipoproteins regulate plasma enzymes involved in lipid metabolism or mediate uptake of lipoproteins in tissues by serving as ligands for cell surface receptors (1). Defects in apolipoprotein structure or biosynthesis may contribute to disorders of the plasma lipid transport system and development of coronary artery disease.

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§ Investigator of the American Heart Association, New York City Affiliate.

¶ Established Investigator of the American Heart Association.

Apolipoprotein CIII (apoCIII)<sup>1</sup> is a major protein constituent of the triglyceride-rich lipoproteins and is present in increased concentration in the plasma of hypertriglyceridemic individuals (2). *In vitro*, apoCIII has been shown to inhibit the hydrolysis of lipids by lipoprotein lipase (3, 4), an enzyme involved in the clearance of triglyceride-rich lipoproteins. ApoCIII has also been found to decrease uptake of triglyceride-rich particles by perfused rat liver (5) and to reduce clearance of modified lipoprotein particles in cebus monkeys (6). ApoCIII is expressed primarily in the liver (5), and although its precise role *in vivo* remains unclear, apoCIII is thought to be involved in the catabolism of triglyceride-rich lipoproteins. Thus, quantitative or qualitative defects in apoCIII expression may contribute to the occurrence of hypertriglyceridemia or other dyslipoproteinemias.

The gene for apoCIII is a member of a dispersed gene family (7, 8) and is tandemly linked to genes for both apoA-I and apoA-IV in the human (9) and rat genomes (10). In this study, we examined the role of 5'-flanking sequences in determining the levels and tissue specificity of apoCIII gene expression. We introduced hybrid genes containing portions of the apoCIII promoter ligated to a reporter chloramphenicol acetyltransferase (CAT) gene into human hepatic and nonhepatic cell lines to map cis-acting DNA elements which regulate apoCIII transcription. Both positive and negative regulatory sequences identified in this manner were further examined for their ability to interact with nuclear proteins from expressing and nonexpressing cell types by electrophoretic mobility shift analysis. Regulatory sequences discovered upstream of the apoCIII gene appear to share homology and/or functional similarity with previously characterized regulatory elements from a variety of other genes, suggesting that similar mechanisms may regulate expression of genes within the apolipoprotein family and beyond.

## EXPERIMENTAL PROCEDURES

**Plasmid Constructions**—The vector pKT (Fig. 2) was constructed by inserting the bacterial chloramphenicol acetyltransferase (CAT) gene into pUC18 between the *Hind*III and *Aat*II sites, adjacent to the intact polylinker. The promoterless CAT gene insert was isolated from the plasmid pSVOCAT (11) as a *Hind*III-*Bam*HI fragment containing the complete CAT coding sequence followed by SV40 splice and polyadenylation sequences. The plasmid pKTSV was produced by insertion of a 366-nucleotide *Kpn*I-*Hind*III fragment from pAW2 (12) into the *Kpn*I and *Hind*III sites in the polylinker of pKT (Fig. 2).

ApoCIII-CAT hybrids were derived from pKT and the previously characterized genomic clone apoA1-6 (13). pKTCIII was constructed by inserting into the *Sma*I site of the pKT polylinker an *Eco*RI-*Pvu*II fragment (generated from a partial *Eco*RI digest of the apoA1-6 clone)

<sup>1</sup> The abbreviations used are: apo, apolipoprotein; bp, base pairs; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CAT, chloramphenicol acetyltransferase; IRE, interferon gene regulatory element.

containing the apoCIII 5'-flanking DNA from -2200 to nucleotide +24 (located in the untranslated first exon). The -1250 construction was made by inserting the *EcoRI-PvuII* fragment extending from -1250 to +24 of the apoCIII gene into the *SmaI* site of pKT. This construction served as the starting material for subsequent constructions. Deletion mutants with endpoints at -685, -210, -110, and -68 were produced by digestion of the -1250 construct with restriction enzymes *StuI*, *SacI*, *MstII*, and *BstXI*, respectively. All other deletions were produced by digestion with *Bal31* nuclease and resulting endpoints determined by dideoxy sequencing as described by Chen and Seeburg (14).

The plasmid pACT (gift of J. Smith) contains a hybrid promoter consisting of a portion of the adenovirus 2 major later promoter (-50 to +33) and 58 bp of SV40 untranslated leader sequence (SV40 map units 5227 to 5171) located immediately upstream of the CAT gene in pKT (Fig. 5B). This plasmid was constructed by inserting a 141-nucleotide *HindIII-PstI* fragment from pSVA677 (15) into the corresponding pKT polylinker sites. In addition, a 499-bp *SalI* fragment from phage  $\lambda$  has been inserted upstream of the hybrid promoter to serve as a spacer between the promoter and test fragments. ApoCIII sequences were inserted at the *SacI* site of the pACT polylinker located upstream of the spacer fragment (see Fig. 5B).

**Cell Culture and DNA Transfection.**—Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (HepG2) or 5% (HeLa) fetal calf serum and plated in 60-mm culture dishes at approximately 25% confluence for DNA transfection. Plasmids were prepared by double banding in cesium chloride and introduced to cells by the calcium phosphate precipitation method (16). To avoid possible interactions between apoCIII regulatory elements and other transcriptional regulatory elements, co-transfection of an internal reference plasmid was not used. Instead, each transfection experiment was repeated a minimum of eight separate times, with at least two different plasmid preparations.

When cells had reached 80% confluence (usually 48 h after transfection) they were harvested, disrupted by freeze-thawing, and protein concentration of extracts were determined. CAT assays were performed on protein equivalents of each sample by the method of Gorman *et al.* (11). CAT activity was quantitated by scintillation counts of spots from chromatograms to determine the proportion of chloramphenicol substrate which had been converted to an acetylated product by the CAT enzyme.

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Analysis.**—Nuclear extracts were prepared from HeLa cells according to Dignam *et al.* (17) and from fresh mouse liver by the method of Gorski *et al.* (18). Nuclear protein-DNA binding reactions (19) were carried out in a volume of 20  $\mu$ l containing 60 mM KCl, 20 mM Hepes (pH 7.9), 4% Ficoll, and 4 mM MgCl<sub>2</sub>. Poly(dIC) was added as a nonspecific competitor, and a typical reaction contained 20,000 cpm (approximately 0.5 ng) of end-labeled DNA with 0.5–5.0  $\mu$ g of extract protein as indicated. After addition of extract, samples were incubated at 20 °C for 30 min and immediately electrophoresed through a native 4% polyacrylamide gel in 0.25  $\times$  TBE (2.2 mM Tris borate, 2.2 mM boric acid, 0.5 mM EDTA). For competition experiments, conditions were as above except that appropriate competitor DNA (as indicated) was included in the reaction mixture prior to addition of extract.

## RESULTS

To facilitate the study of apoCIII gene expression, we extended the previously reported sequence to include 821 base pairs upstream of the transcription initiation site (Fig. 1). A portion of the apoCIII gene from approximately -2200 to +24 (relative to the start site of transcription) was cloned immediately upstream of the CAT gene coding sequences in the plasmid pKT (*pKTCIII*, Fig. 2A). To identify DNA sequence elements important for transcription, a series of deletion mutants extending from -2200 toward the start site of transcription was constructed (Fig. 2B). These mutant constructions were introduced into both HepG2 (human hepatoma) and HeLa (human epithelial) cells by the calcium phosphate co-precipitation method and transient expression of CAT enzymatic activity measured. In each experiment, replicate Petri dishes of cells were also transfected with pKT as a negative control, or with pKTSV (an SV40 early promoter/CAT construct, Fig. 2A) as a positive control. In addition to

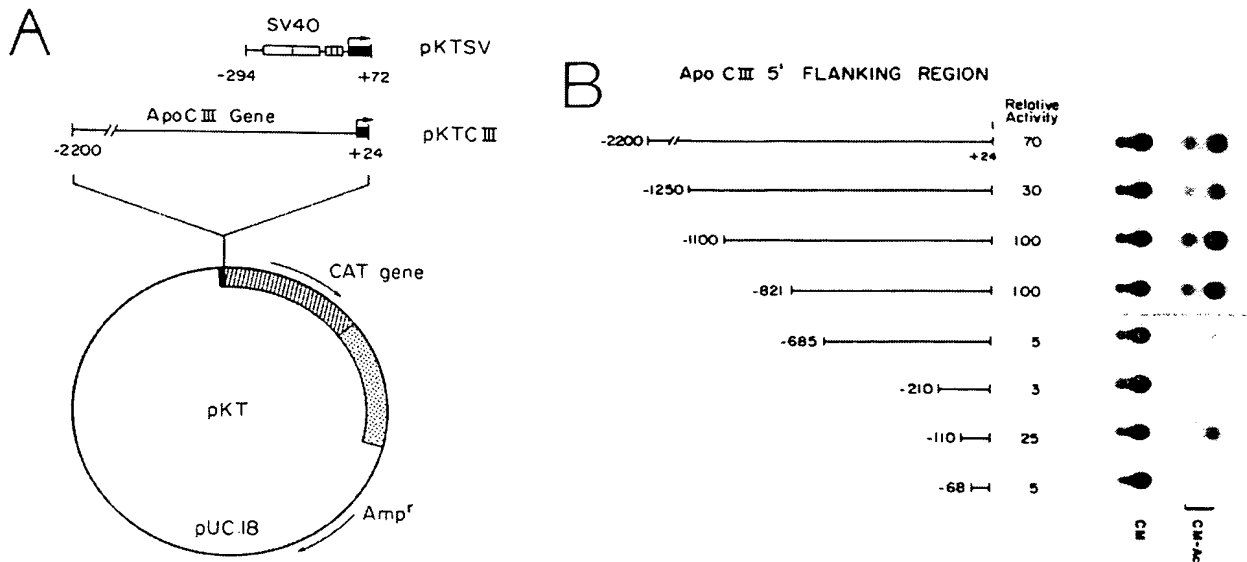
-821	GCTCTGCCCGAGCCCACTGACGAACCCAGCAAGCTCAACAGAGAATCACTCTGGTG	
-761	GGGCTCTCTCCCGAGGGATGTTATGAGTGGCTCAGAGGGCAAAATAGGAGCGCTGCTG	
-701	GAGGGAGGGGCAAGCCCTCGGGCTCTGAGGGCCCTTGGCGCTTCTCCACCAACCGCTGCC	
-641	CTACACTCAGGGGAGGGGGCGGTGGGGCAGACAGGCTGGGGGGCGGTGGGGCGCTCTCTG	
-581	GGTGACGAGCACTCGGCTGGCTGGATTGAACCCAGAGATGGAGGTCTCGGACGGCGCTG	
-521	TCAGCTCAGGCGCTGTAACGAGGCGTTGGCAGCCACTGATGCCGGCTCTTCTGCGCTTTA	
-461	CTCCAAACAGCCCGCCAGCCGAGCCAGCCACTTGTCTCAAGTCTGAAGAAGCGCGCTCAC	
-401	CCCTCTACTCCAGGCTGTGTTAGGCGCTTGGCGCTCTGCGAGGAGCGGCGCTCAAAATTCG	
-341	ACTGTCAACCGCTGAGATGGGGCGGAGCGCTGGCGCTATCTCAAGCCATTCCTCTCTCA	
-281	CCAGGCTCTCCCTGGGAGCCCACTGACGTAGCAAGGAATGAGGCTCCCGAGGCCACGCC	
	*****	
	AAGCGTTCTCGGCTGACTCTTTCTGCTCTCAGCAGCTCTCATCGAGGAG	R
-221	CAGTTCCTGAGCTCATCTGGGCTGCGGCTGGCGGAGCAGCGGTGGACTCAGTCTCC	
	*****	
	CTCAGTACGTATCTCTCTGCGCTGGCGAGCTGCAAGTCAACGCTCTCGGCAATCTCT	R
-161	TAGCGATTTCGCAACTC---TCGGCGCGGCTTGC-TGCATCTGACACCGCTGCGCTCAGCG	
	*****	
	GAGCGATTTCCTCACTCTCTGCGGAGCTGGCTGCAATGGCGTCCCTCGCTCGGCTCTGCG	R
-105	CCTCATCTCCACTGCTCAGCAGCTGACCTTGGCCAGCGCGCTGGG---TC-CT-CAGTG	
	*****	
	TGTC---CACTGTTGAGGAGCTGACCTTGACAGGCTCAGTGGCGCTCTGCTGCGCGCG	R
-50	CCTGCTCGGCTGGAGATGATATAAAACAGCTCAGAACCGCTCCTGCTCTCT	
	*****	
	TGTCGATCTCGGAGCAATATAAAACAGATCAG-AGCGTCCGCGCTTGC	R

FIG. 1. Nucleotide sequence of human apoCIII gene 5'-flanking region and alignment with available sequence data for corresponding region of rat apoCIII gene. The sequence presented for human apoCIII confirms that previously reported (to position -192, see Ref. 38) and extends to 821 nucleotides upstream of the start site of transcription (indicated by arrows). The rat sequence (R) (10), has been aligned for maximum homology. Numbers refer to nucleotide positions of the human sequence with respect to the start site of transcription. An asterisk indicated identity between human and rat sequence.

CAT activity determinations in selected experiments, correct mRNA initiation was demonstrated by primer extension of RNA isolated from cells transfected with the pKTCIII construction and many of the deletion mutants (data not shown). Primer extension also demonstrated that the relative levels of CAT mRNA for the mutants tested parallels the amount of CAT enzyme detected.

The positive control pKTSV was expressed in both HepG2 and HeLa cells, whereas the pKT plasmid was expressed in neither cell type. The apoCIII-CAT hybrids were expressed in HepG2 cells at varying levels depending on the amount of apoCIII 5'-flanking DNA present (see below), while expression of these hybrids in HeLa cells did not exceed the background level for pKT. This complete inactivity of apoCIII-CAT hybrids in HeLa cells was not due to a lower transfection efficiency for these cells, as expression directed by the viral promoter of pKTSV was actually higher in HeLa than in HepG2 cells. In the HepG2 cells, successive deletion of apoCIII upstream sequences revealed that multiple cis-acting DNA sequence elements contribute to the regulation of apoCIII transcription. Successive deletions (see Fig. 2B), extending from -2200 to -821, revealed a relatively weak positive element (-2200 to -1250) and a weak negative element (-1250 to -1100) each of which modulates expression by 2–3-fold. Between -821 and -685, there appears to be a very strong positive element, the deletion of which decreases expression 20-fold. Interestingly, between -220 and -110 there is a strong negative element which when deleted resulted in an 8-fold increase in expression. Finally, between -110 and -68 there is another positive element which has at least a 5-fold effect on apoCIII gene transcription. We have characterized in detail these last three regions.

**ApoCIII Proximal Positive Element (-110 to -68)—To**



**FIG. 2. Structure and expression of apoCIII-CAT hybrid constructions.** A, structure of plasmid vector pKT and derivatives. pKT (see "Experimental Procedures") contains the entire CAT gene coding sequence (hatched region) and SV40 splice site and polyadenylation signal (stippled region) inserted into the pUC18 polylinker (solid region). pKTCIII contains human apoCIII gene sequences from -2200 to +24 (relative to the start site of transcription) ligated into the polylinker of pKT. pKTSV contains SV40 early promoter sequences, including the two 72-bp and three 21-bp repeats (open boxes), inserted into the pKT polylinker. Solid boxes and arrows in pKTCIII and pKTSV represent exonic sequences and transcription start sites, respectively. B, stimulation of CAT gene expression by apoCIII 5'-flanking DNA sequences. A series of plasmids containing deletions in the 5'-flanking DNA were constructed from pKTCIII and their activity in HepG2 cells determined as described under "Experimental Procedures." Activities are expressed relative to that achieved with the -821 construction and represent the mean of at least eight separate transfections; variation between experiments in expression of apoCIII-CAT hybrids relative to pKTSV did not exceed 20%. A representative CAT assay is shown with unreacted chloramphenicol (CM) and acetylated products (CM-Ac) indicated. No expression was observed in HeLa cells.

characterize the proximal positive element, the region between -110 and -68, was subdivided by a series of small deletions extending toward the start-site of transcription from the -110 5' end point. The end points and expression levels (relative to the -110 constructions) of these mutants in HepG2 cells were: -110 (100%); -96 (44%); -82 (44%); -77 (24%); and -68 (16%). These results suggest that the proximal positive element consists of two separate regions (-110 to -96 and -82 to -77) which contribute additively to the level of apoCIII gene transcription in HepG2 cells. These results are supported by the observation that these two regions share strong sequence homology to portions of the rat apoCIII gene, other apolipoprotein genes (B, A-I, A-IV), and another liver expressed gene,  $\alpha_1$ -antitrypsin (Fig. 1, Table I).

We have also demonstrated a difference in binding pattern of nuclear proteins from liver and HeLa cells to the -82 to -77 DNA region. Utilizing the electrophoretic mobility shift assay we found that only a single protein from a liver nuclear extract bound to a radiolabeled apoCIII gene fragment extending from -110 to +24 (Fig. 3, lane e). To further localize the region to which this protein binds, similar experiments were performed with radiolabeled apoCIII gene fragments -96 to +24 (lane f), -82 to +24 (lane g), and -77 to +24 (lane h). The liver protein failed to bind when sequences between -82 and -77 were deleted (Fig. 3, compare lanes e-g to lane h) or when an excess of unlabeled homologous DNA fragment was included as a competitor (not shown). We have designated the liver protein that binds to this transcriptionally active region liver protein-1 (LP-1).

When the HeLa nuclear extract was used, no protein band with the same electrophoretic mobility as LP-1 is detected,

although several HeLa-specific bands are visible (Fig. 3, lane a). Interestingly, one of these HeLa-specific proteins (Fig. 3, lanes a-c, arrow), which is only visible when high concentrations of nuclear extract are used, also does not bind when sequences between -82 and -77 are deleted (Fig. 3, compare lanes a-c to lane d). Thus, this HeLa protein and LP-1, while not identical proteins, require similar sequences for binding, suggesting that the cell type-specific activity of this region is due to differing activities of the two proteins.

**ApoCIII Negative Element (-210 to -110)**—Further characterization of the negative element between -210 and -110 was aided by the identification of a DNA sequence homologous to a known regulatory element. The reverse orientation of the human apoCIII sequence from -160 to -149 is almost identical to a portion of the  $\beta$ -interferon gene regulatory element (Ref. 20, see Table I). In addition, although the human and rat apoCIII genes show very little homology between -210 and -110, the same putative regulatory region is conserved in 15 out of 16 positions between -160 and -145 (Fig. 1).

To verify the importance of this region, an eight-nucleotide linker was inserted at a restriction site between position -160 and -159 (construction -210A, Fig. 4A). In addition, *Bal*31 digestion was carried out at this restriction site to produce an internal deletion of sequences from -175 to -122 (construction -210B, Fig. 4A). Activity of the wild-type -210 construction (Fig. 2) was then compared with the -210A, -210B, and -110 constructions in transient expression assays in both HepG2 and HeLa cells (Fig. 4A). None of the mutations in the negative element resulted in apoCIII expression in HeLa cells. However, in HepG2 cells, the linker insertion resulted

TABLE I

## Homologies of apoCIII 5'-flanking DNA with other gene regulatory elements

Upper case letters indicate homology between the particular apoCIII regulatory element and heterologous gene sequence. Sequence coordinates indicate position with respect to the start site of transcription and do not necessarily correlate with sequence coordinates presented in original references.

Sequence	Name	Homology	Reference
<b>ApoCIII proximal element (-112/-102)</b>			
CTCAGGCCCT	ApoCIII -112/-103		
CTCtGG-TCT	Rat apoCIII -108/-100	7/10	10
gTCAGGCCCG	Human apoB -94/-85	8/10	37
CTgA-aCCCT	Human apoA-I -236/-238	7/10	13
CTgAG-CCCT	Human apoA-IV -180/-172	8/10	22
CTtAGcCCCT	Human $\alpha_1$ -antitrypsin -115/-106	8/10	23
<b>ApoCIII proximal element (-84/-76)</b>			
GGTGACCTT	ApoCIII -84/-76		
GGTGACCTT	Rat apoCIII -87/-79	9/9	10
GGcGcCCCT	Human apoB -81/-73	7/9	37
GcTGAtCCCT	Human apoA-I -159/-150	8/10	13
tGTcACCTT	Human apoA-IV -108/-100	7/9	22
GGTGACCTT	Human $\alpha_1$ -antitrypsin -84/-76	9/9	23
<b>ApoCIII negative element</b>			
GAGTTGGGAAATCCCT	ApoCIII -145/-160		
GAGTTGaGAAATCCCT	Rat apoCIII -147/-162	15/16	10
TGGGAAATcCCT	Human $\beta$ -interferon IRE -65/-54	11/12	20
<b>ApoCIII distal positive element</b>			
CCCCACTGAGGAACC	apoCIII -807/-794		
TGtGGAA	SV40 enhancer core	6/7	21
CCCCAtTGA	Cytomegalovirus 19-bp repeat	8/9	21

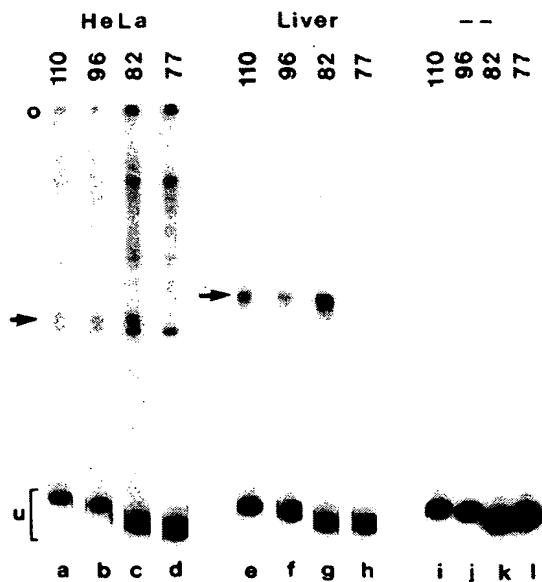


FIG. 3. Protein factors that bind to the apoCIII proximal positive element. Labeled DNA fragments containing various portions of the apoCIII promoter were incubated with nuclear extracts prepared from HeLa (lanes a-d) (6.7  $\mu$ g of protein/reaction) or mouse liver (lanes e-h) (2.5  $\mu$ g of protein/reaction) cells, and specific DNA-protein complexes were analyzed by the electrophoretic mobility shift assay (see "Experimental Procedures"). The DNA fragments contained sequences from +24 (relative to the start site of transcription) to -110 (lanes a, e, and i); -96 (lanes b, f, and j); -82 (lanes c, g, and k); and -77 (lanes d, h, and l). The origin (o) and unbound DNA (u) are indicated. Arrows indicate DNA-protein complexes that require sequences from -82 to -77 for binding. Lanes i-l are control reactions that do not contain extract.

in a 2-fold increase and the deletion a 3-fold increase in expression when compared with the -210 wild-type construction. In neither case, therefore, did expression levels equal that of the -110 construction. This may indicate incomplete disruption of the negative element by the linker insertion (-210A) and the deletion (-210B). To further explore the properties of the apoCIII gene negative element, the DNA fragment from -210 to -110 was inserted in both orientations into the pKTSV plasmid either immediately upstream of the SV40 72-bp repeat enhancer or between the enhancer and the early promoter (21-bp repeat) sequences (see Fig. 2A). In none of these constructions did the apoCIII gene negative element affect transcription.

Utilizing the gel mobility shift assay, we have shown that nuclear extracts prepared from HepG2 and HeLa cells contain proteins that bind specifically to the apoCIII negative region. Radiolabeled apoCIII gene fragments isolated from the wild-type and the -210A constructions (extending from -210 to +24) show similar binding patterns in the liver cell nuclear extracts (Fig. 4B, compare lanes d-f to lanes g-i). However, the binding pattern of the analogous radiolabeled fragment from the -210B mutant is missing two of the protein bands from the liver extract (Fig. 4B, compare lanes d-f to lanes m-o) and one from the HeLa extract (Fig. 4B, compare lanes a-c to lanes j-l). The mobility of this HeLa band corresponds to the mobility of one of the two liver bands. These results demonstrate that one protein unique to liver and one protein common to both liver and HeLa cells binds to the negative acting region between -175 and -122.

These results were confirmed with the complementary experiment in which binding to the radiolabeled wild-type -210 fragment was competed by unlabeled -210, -210A, and -210B templates. Both the wild-type and the -210A fragment competed for binding of all proteins retained by the -210 fragment. In contrast, the -210B fragment failed to compete for binding of these two liver proteins (data not shown). Thus, deletion of sequences homologous to the  $\beta$ -interferon gene regulatory element both diminishes the effect of the negative

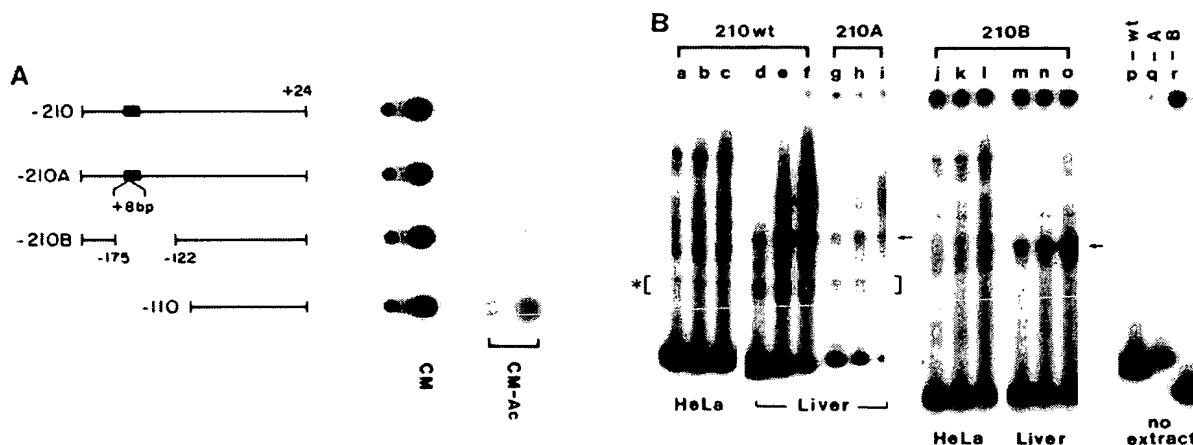


FIG. 4. ApoCIII negative element: effect of mutation on expression and protein binding. A, mutations were produced by insertion (-210A) or deletion (-210B) of sequences in the region homologous to the  $\beta$ -interferon gene regulatory element (represented by solid bar). Stimulation of CAT gene expression in HepG2 cells by each of these mutants (compared to wild-type -210 and -110 constructions) is shown at right, with unreacted chloramphenicol (CM) and acetylated products (CM-Ac) indicated. No expression was observed in HeLa cells. B, labeled DNA fragments containing the apoCIII promoter segments from -210 to +24 (with respect to the start site of transcription) were incubated with HeLa or liver nuclear extracts (as indicated), and specific DNA-protein complexes were analyzed by the electrophoretic mobility shift assay (see "Experimental Procedures"). HeLa protein amounts per reaction were 3.3  $\mu$ g (lanes a and j), 6.7  $\mu$ g (lanes b and k), and 10  $\mu$ g (lanes c and l). Liver protein amounts per reaction were 2.5  $\mu$ g (lanes d, g, and m), 5  $\mu$ g (lanes e, h, and n), and 7.5  $\mu$ g (lanes f, i, and o). Specific labeled templates used in each experiment are indicated at the top of each lane and shown in A. The asterisk indicates protein-DNA complexes that disappear when sequences from -175 to -122 are deleted. Arrows indicate the complex identified as LP-1 (see Fig. 3 and text).

element on levels of transcription and eliminates binding of specific protein factors.

**Distal Positive Element (-821 to -685)**—To characterize the distal positive element between -821 and -685, intermediate deletions were made extending from the -821 end point toward position -685. The endpoints and expression levels (relative to -821) of these constructions in HepG2 cells are shown in Fig. 5A. The DNA element necessary for high levels of expression appears to reside within the 49 bp between -821 and -772. To test positional effects of the -821 to -685 region within its own promoter environment, segments from -685 to -210 were deleted from the apoCIII promoter. This placed the apoCIII distal positive element 210 nucleotides from the start site of transcription. In this position, it stimulated transcription in HepG2 cells by more than an order of magnitude relative to the -210 construction (Fig. 5A). The activity of this construction was still less than for the intact -821 construction, however, indicating the presence of an additional positive modulator in the region between -685 and -210. Portions of the distal positive region are homologous to consensus sequences of the SV40 and cytomegalovirus enhancers (see "Discussion" and Table I).

To determine if the apoCIII gene distal positive element functions in a manner similar to other enhancer elements, the DNA fragment from -821 to -685 was cloned upstream from the heterologous adenovirus-2 major late promoter (Ad2MLP). The apoCIII gene fragment was placed in both orientations at approximately -600 in the AdMLP-CAT construction pACT (see Fig. 5B). This distance is similar to its position upstream of the apoCIII promoter. The apoCIII distal positive element in these constructions stimulates transcription in both forward and reverse orientations in HepG2 cells (Fig. 5B). The maximum degree of stimulation in HepG2 cells is 4-fold, which is significantly less than the 20-fold effect observed when this element is deleted from the apoCIII pro-

moter (Fig. 3B). The distal positive element did not stimulate expression in HeLa cells when placed upstream of either the heterologous or the apoCIII promoter. Thus, the apoCIII distal positive element demonstrates at least some of the characteristics of a classical viral enhancer (21) and, in addition, appears to be tissue-specific in its activity.

Mobility shift assays performed with the radiolabeled -821 to -685 DNA fragment demonstrated specific binding of proteins from hepatic but not HeLa cell nuclear extracts (Fig. 6). The two shifted bands which result from binding of liver proteins are completely lost when unlabeled -821 to -685 fragment is added as competitor (Fig. 6, compare lane b to lane d). Competition with a fragment of DNA containing the SV40 enhancer, however, does not diminish binding of these proteins (Fig. 6, compare lane b to lane e). None of the shifted bands produced with the HeLa extract represent specific binding as they are not competed by addition of the unlabeled apoCIII fragment (Fig. 6, compare lane b to lanes d and e). These results suggest that the lack of activity of this element in HeLa cells may be due to the absence in this cell type of positive acting protein factors that bind to the element.

#### DISCUSSION

**Positive and Negative Cis-acting Elements Regulate ApoCIII Expression and Share Homology with Regulatory Sequences from Other Genes**—By introducing a series of apoCIII promoter deletion mutants into tissue culture cells, we have identified several cis-acting DNA sequence elements which modulate the expression of the apoCIII gene. To determine how these sequence elements contribute to tissue specific expression of the apoCIII gene, we measured the transcriptional activity of these mutants in hepatoma (HepG2) and epithelial carcinoma (HeLa) cell lines. In the 2 kilobases upstream of the apoCIII gene transcriptional start site, we characterized two positive elements and a single negative

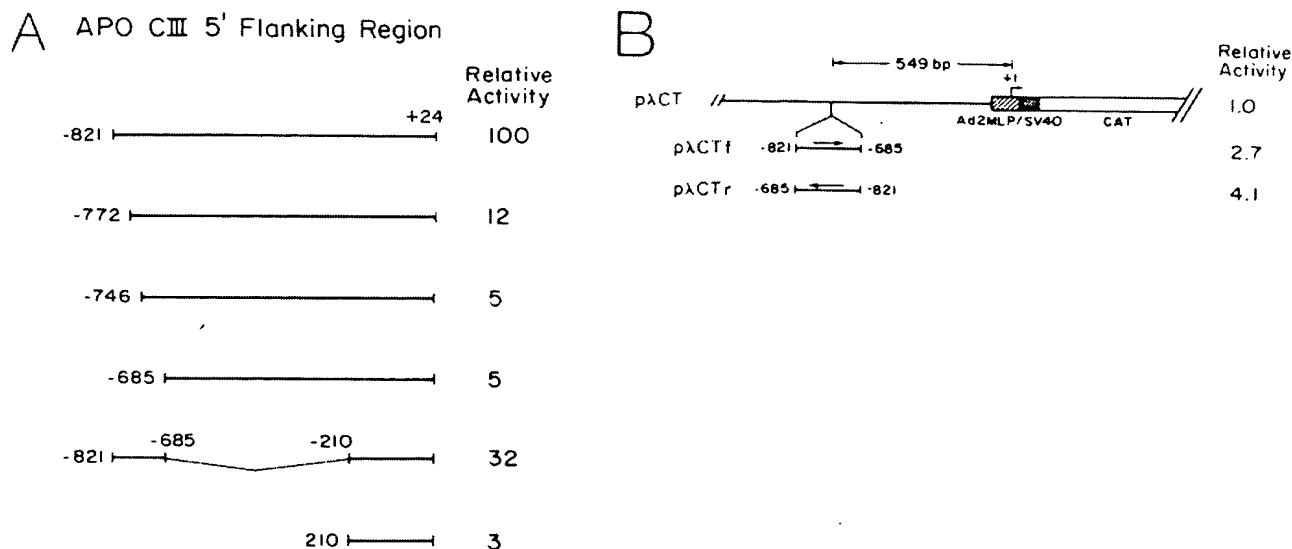


FIG. 5. ApoCIII distal positive element deletion analysis and stimulation of expression of a heterologous promoter. A, a series of plasmids containing 5' deletions between -821 and -685 were produced with *Bal31* nuclease digestion. In a separate construction, the -821 to -685 region was inserted immediately upstream of the proximal 210 bp of the apoCIII promoter. Activities of these constructions in HepG2 cells were determined as described under "Experimental Procedures" and are expressed relative to the -821 construction. No expression was observed in HeLa cells. B, ApoCIII 5'-flanking DNA from -821 to -685 was inserted in both orientations upstream of a heterologous promoter in the vector pλCT (described under "Experimental Procedures"). pλCT contains the adenovirus 2 major late promoter (hatched box) and untranslated leader sequences from SV40 (cross-hatched box) located upstream of the CAT gene (open box). ApoCIII sequences were inserted 549 bp upstream of the major late transcription start site (designated +1) in forward (pλCT-f) or reverse (pλCT-r) orientation. Activity of these constructions in HepG2 cells were determined as described under "Experimental Procedures" and are expressed relative to the basal level of pλCT activity. No expression above the pλCT basal level was observed in HeLa cells.

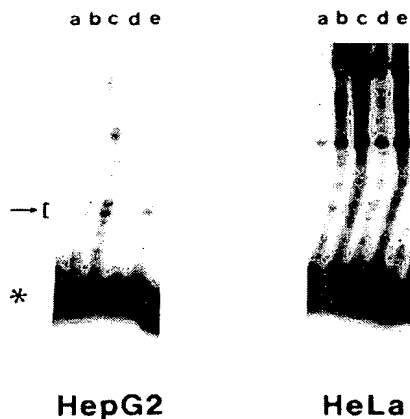


FIG. 6. Protein factors that bind to the apoCIII distal positive element. A DNA fragment containing apoCIII sequences from -821 to -685 was radiolabeled and assayed for protein binding activity in the electrophoretic mobility shift assay with nuclear extracts prepared from HepG2 and HeLa cells. Lanes a-c, protein binding pattern with increasing amounts of nuclear extract (0.5, 1.0, and 2.0 μg of protein); lanes d, with 1.0 μg of protein extract and 50-fold molar excess of unlabeled -821 to -685 apoCIII fragment; lanes e, with 1.0 μg of protein extract and 100-fold molar excess of unlabeled SV40 72-bp enhancer fragment. Bracket indicates retained bands from HepG2 extract which are specifically competed by the apoCIII -821 to -685 fragment. The asterisk indicates position of unbound DNA fragment.

element. The proximal positive element is within 110 bp of the transcription start site and contains two active areas (-110 to -96 and -82 to -77). The negative element is slightly further upstream between -210 and -110. The distal positive element is much further upstream between -821 and -772. Each of these elements functions in HepG2 but not HeLa cells.

DNA sequences which influence expression of the apoCIII gene and bind specific proteins from nuclear extracts are highly conserved between the human and rat genes (Fig. 1) and share homologies with known regulatory elements from other genes (Table I). Sequences that resemble the apoCIII proximal positive elements (-110 to -96 and -82 to -77) appear upstream of genes for human apolipoproteins A-I, A-IV, and B. In one case (A-IV), the region -127 to -60, which contains a sequence homologous to the apoCIII proximal positive element, has been shown to be a positive regulatory region (22). In addition, homologous regions upstream of the human apoB gene have an activity like the apoCIII positive elements in modulating levels of expression from an apoB-CAT hybrid gene.<sup>2</sup> An interesting possibility is that these elements represent a common functional motif in the regulation of apolipoprotein gene expression.

In addition to other apolipoprotein genes, the apoCIII regulatory elements share homology with defined regulatory elements from less closely related genes and viral enhancers (Table I). A striking degree of homology exists in both sequence and spatial arrangement of the apoCIII proximal positive elements and sequences upstream of the gene for human  $\alpha_1$ -antitrypsin. Like apoCIII,  $\alpha_1$ -antitrypsin is prefer-

<sup>2</sup> H. Das, T. Leff, and J. L. Breslow, unpublished observations.

entially expressed in liver, and transfected  $\alpha_1$ -antitrypsin minigenes are expressed in HepG2 but not in HeLa cells (23). These findings suggest that at least some liver-specific genes may share mechanisms of regulation of transcription. A demonstration that the same protein factors bind to the common sequences upstream of these two genes would lend further support to this possibility.

Negative cis-acting DNA regions have been described only recently in mammalian genes. Initial reports of the rat  $\alpha$ -fetoprotein (24), rat insulin 1 (25, 26), and rat growth hormone genes (27) did not identify specific sequences which confer the negative activity. However, Goodburn *et al.* (20) identified and sequenced an inducible enhancer upstream of the human  $\beta$ -interferon gene (the interferon gene regulatory element, IRE) which is under negative control. The negative region upstream of the apoCIII gene, in reverse orientation, is highly homologous to a portion of the IRE with 11 out of 12 matches (see Table I). A negative-acting element upstream of the pea rbcS-3A gene, which shares homology with the IRE, has also been described (28). Negative regulation may be a general mechanism controlling eukaryotic gene transcription. It is intriguing that the three sequences identified for these negative elements appear to be closely related. The only other identified sequence homology to the human apoCIII negative element was in the equivalent region of the rat apoCIII gene (Table I). The element is conserved in 15 of 16 positions in the rat apoCIII gene, a sharp contrast to the overall lack of homology between the human and rat sequences extending beyond the first 100 nucleotides upstream of the gene (see Fig. 1).

Several of these negative-acting sequences exert an effect only under particular conditions. The  $\beta$ -interferon regulatory element acts only when the interferon gene is in an uninduced state (20). The negative element upstream of the light-inducible pea rbcS-3A gene influences expression only in dark-adapted plants (28). The rat growth hormone negative region, known as the thyroid inhibitory element, represses transcription only after treatment of cells with thyroid hormone (27). It would be interesting to determine if, like many of the negative elements which have been described, the activity of the apoCIII negative element is regulated by physiological conditions in the cell and, if so, what implication this may have for the role of apoCIII in lipid metabolism.

In the interferon and rbcS-3A genes, the negative elements are actually part of more complex regulatory elements which behave as inducible enhancers. Perhaps related to their enhancer-like characteristic, the negative regions from these two genes are able to suppress transcription from heterologous promoters as well (20, 28). In contrast, the apoCIII negative element does not appear to suppress expression from the heterologous SV40 promoter. This observation is similar to the rat hormone gene thyroid inhibitor element (27) which does not appear to suppress the thymidine kinase gene when inserted into its promoter. It is possible that these differences are a function of the specific heterologous promoters employed or that the apoCIII and rat hormone thyroid inhibitory elements behave in a truly distinct manner from the enhancer-like negative elements of the interferon and rbcS-3A genes.

The distal positive element (-821 to -772) is essential for high levels of expression in HepG2 cells. It increases expression by more than an order of magnitude both in its native configuration and when placed directly upstream of the first 210 nucleotides of the apoCIII promoter. Furthermore, a 136-nucleotide long fragment (-821 to -685) containing the element can enhance transcription from the adenovirus major late promoter when inserted upstream in either orientation.

The ability to stimulate transcription from a heterologous promoter in either orientation and from a distance of several hundred nucleotides is characteristic of enhancers found associated with many viral and cellular genes (21). The apoCIII distal positive element shares homology with consensus sequences for enhancers from SV40 and cytomegalovirus (Table I). No homology was detected between the apoCIII element and an enhancer which has been discovered upstream of the human apoA-II gene (29). The apoCIII positive element does not enhance expression in HeLa cells even when placed upstream of a heterologous promoter. This is similar to many cellular enhancers (30, 31) including the one found upstream of the apoA-II gene that stimulates transcription only in restricted cell types (29). Thus, the CIII positive element is best described as a cell-type specific enhancer element.

**Role of Trans-acting Factors in Determining Cell Type Specificity of ApoCIII Expression**—All transcriptionally active apoCIII-CAT hybrids were expressed exclusively in HepG2 cells. Hepatocyte-specific expression could result from the presence of positive factors in HepG2 cells that are missing in HeLa cells or, conversely, negative factors present in HeLa cells but not HepG2 cells. In the case of apoCIII, expression and protein binding data indicate that cell type-specific expression is governed by hepatic-specific positive elements. In no case does deletion of apoCIII sequences result in detectable expression in HeLa cells, as would be expected with the mutation of a crucial HeLa-specific negative element. In addition, the distal positive element increases transcription from a heterologous promoter only in HepG2 cells, while the negative element is not active with a heterologous promoter in HeLa cells. Furthermore, HeLa nuclear extracts do not appear to contain proteins that bind specifically to the distal positive element, and although binding of a HeLa protein does occur to the proximal positive element, this protein is of low abundance and/or affinity and of a different mobility than the liver protein LP-1 (see Fig. 4). This HeLa protein may represent either a modified (inactive) form of the liver protein or an entirely different protein which binds to the same sequence. A similar phenomenon has been described for other tissue-specific regulatory elements including the immunoglobulin octamer sequence (32, 33) and the chicken  $\alpha$ -actin promoter (34). In both of these cases, the same protein binding sequence interacts with different protein species in extracts prepared from expressing and nonexpressing cell types.

**Similarities Between ApoCIII Gene Expression and That of Other Genes Expressed in the Liver**—Recently, expression studies of two other apolipoprotein genes, apoA-II and apoA-IV, have been reported. Initial studies of human and rat apoA-IV expression demonstrate that sequences located within 900 nucleotides of the start site are required for maximum levels of expression and affect cell type-specific expression (22). Studies of human apoA-II gene expression have revealed an upstream enhancer element that confers tissue-specific expression on this gene (29). Like the apoCIII upstream positive element described here, the apoA-II enhancer is centered at approximately 800 nucleotides upstream of the gene and exhibits cell type restricted activity. Unlike the apoA-II enhancer, however, the apoCIII positive element is not absolutely required for transcription from the apoCIII promoter. It remains to be seen whether the regulatory mechanisms employed in the expression of the apoCIII or apoA-II genes are shared by other apolipoprotein genes and what implications such mechanisms may have on their respective roles in lipid metabolism.

The tissue-specific activity of both distal and proximal



positive elements of the apoCIII gene invite comparison with the mouse  $\alpha$ -fetoprotein gene. In transient expression assays (35) and in transgenic mice (36), cell type specificity of  $\alpha$ -fetoprotein expression is mediated by a combination of regulatory elements which includes three enhancers located several kilobases upstream, and the promoter region located between -85 and -52 bp upstream from the transcription start site. Thus, the apoCIII gene and other genes demonstrating tissue-specific expression appear to be under the control of multiple regulatory elements. The extent to which the regulatory elements from these diverse genes share specific sequences or interact with similar trans-acting proteins awaits further study.

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